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MINIREVIEW

Bacterial Endotoxins: Extraordinary Lipids That Activate Eucaryotic Signal Transduction

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INTRODUCTION

Over 100 years ago, Pfeiffer and Centanni described heat-stable, nonsecreted toxin(s) in gram-negative bacteria that caused fever and pathology when injected into animals (53). Unlike heat-labile protein toxins, such as diptheria and tetanus toxins, the cell-associated toxins of gram-negative bacteria (endotoxins) consisted of carbohydrates and lipids (36, 53) and are also termed lipopolysaccharides (LPS) (see Fig. 1). Uncertainties about the chemical nature of endotoxins lingered until 1983, when the structure of the lipid A anchor of LPS (see Fig. 1 and 2) was elucidated (36, 46, 53), and chemically synthesized lipid A was shown to possess the biological activities of endotoxins (36, 46, 53).

The long delay in establishing the identity of the active component of endotoxins occurred because the structure of lipid A is complex (29, 36, 46) and differs from those of all other lipids (45). The approximate composition of lipid A was recognized 40 years ago (36, 53), but its biosynthesis and pharmacology could not be studied without a complete understanding of its covalent chemistry (46).

In 1983, just as several laboratories were close to defining the structure of lipid A (54), my group characterized a novel glucosamine-derived phospholipid (58), previously observed in certain phosphatidylglycerol-deficient mutants of *Escherichia coli* (40). Together with Laurens Anderson and Kuni Takayama at the University of Wisconsin (58), we showed that this material, termed lipid X, was a 2,3-diacylglucosamine 1-phosphate (see Fig. 2). The discovery of a monosaccharide substructure of lipid A provided an important clue to elucidating the sites of acylation on lipid A (40, 54, 58) and the enzymatic pathway for lipid A biosynthesis (1, 14, 46, 50). The functional connection between lipid X accumulation and phosphatidylglycerol deficiency remains unknown (39, 40).

In this minireview, I summarize current knowledge of the enzymatic synthesis of lipid A, most of which is derived from studies with *E. coli* extracts (46, 65). I also provide a brief overview of the mechanisms by which lipid A activates signal transduction in animal cells.

LIPID A COMPARED WITH GLYCEROPHOSPHOLIPIDS

The minimal LPS required for growth of gram-negative bacteria, termed Re LPS (36, 38, 46), occurs in mutants lacking heptose (see Fig. 1). Re LPS consists of lipid A and two 3-deoxy-D-*manno*-octulosonic acid (KDO) moieties (see Fig. 2) (36, 46). Re LPS possesses 24 chiral centers, whereas classical glycerophospholipids contain one or two. Lipid A is therefore an "information-rich" molecule, with many possibilities for specific recognition by procaryotic and eucaryotic proteins. The acyl moieties of glycerophospholipids are often 2 to 6 carbons longer than those attached to lipid A (45, 46), but they are not hydroxylated (45, 47). The acyl moieties of lipid A generally do not contain double bonds (36, 46). There are $\sim 10^6$ lipid A molecules and $\sim 10^7$ glycerophospholipids in a cell (24, 45), arranged as shown in Fig. 1. The reasons why lipid A and KDO are required for growth (46) are not known.

The LPS molecules of wild-type cells are further glycosylated with 6 to 8 additional sugars that constitute the nonrepeating core domain and ~1 to 50 O-antigen repeats (Fig. 1) (36, 46). The latter are often tetrasaccharides (36, 46) characterized by the presence of deoxy- and dideoxyhexoses.

DISCOVERY OF THE LIPID A PATHWAY

Recognition that lipid X was equivalent to the reducing end glucosamine of lipid A (Fig. 2) (58) suggested that it must be a precursor or breakdown product (50, 58). The discovery in 1983 that KDO was attached at position 6′ on lipid A (Fig. 2) (54) and that position 3′ (Fig. 2) was acylated with *R*-3-hydroxymyristate revealed that the nonreducing end glucosamine of lipid A was acylated with *R*-3-hydroxymyristate (Fig. 2) in the same places as the reducing end unit. Accordingly, we decided to search for a pathway by which the 2,3-diacylglucosamine moiety of lipid X would be incorporated into both sugars of the lipid A disaccharide (14, 50).

As an initial approach, Ray et al. (50) synthesized UDP-2,3-diacylglucosamine (Fig. 3), a putative nucleotide derivative of lipid X. When UDP-2,3-diacylglucosamine and lipid X were incubated with a crude extract of wild-type *E. coli*, efficient formation of a new, more hydrophobic product was observed (50). Isolation and physical analysis demonstrated that this material consisted of two glucosamines, four *R*-3-hydroxymyristates, and one anomeric phosphate (Fig. 3) (50). The presence of a novel disaccharide synthase in cell extracts, capable of generating the β, 1′-6 linkage of lipid A, demonstrated that lipid X and its UDP derivative were precursors (50). Neither compound had been discovered earlier, because each represents less than 0.01% of the total lipid of wild-type *E. coli* (14, 45).

FORMATION OF UDP-2,3-DIACYLGLUCOSAMINE

Studies with extracts of *E. coli* have shown that UDP-GlcNAc (but not UDP-GlcN or GlcN-1-P) can be acylated at position 3 (Fig. 4) (1, 3). *E. coli* UDP-GlcNAc *O*-acyltransferase is selective for *R*-3-hydroxymyristate, consistent with the composition of *E. coli* lipid A (2, 3). This enzyme has an absolute requirement for an acyl carrier protein (ACP) thioester and for the *R*-3-OH moiety (2, 3), as myristoyl-ACP is not a substrate. The equilibrium constant for O acylation

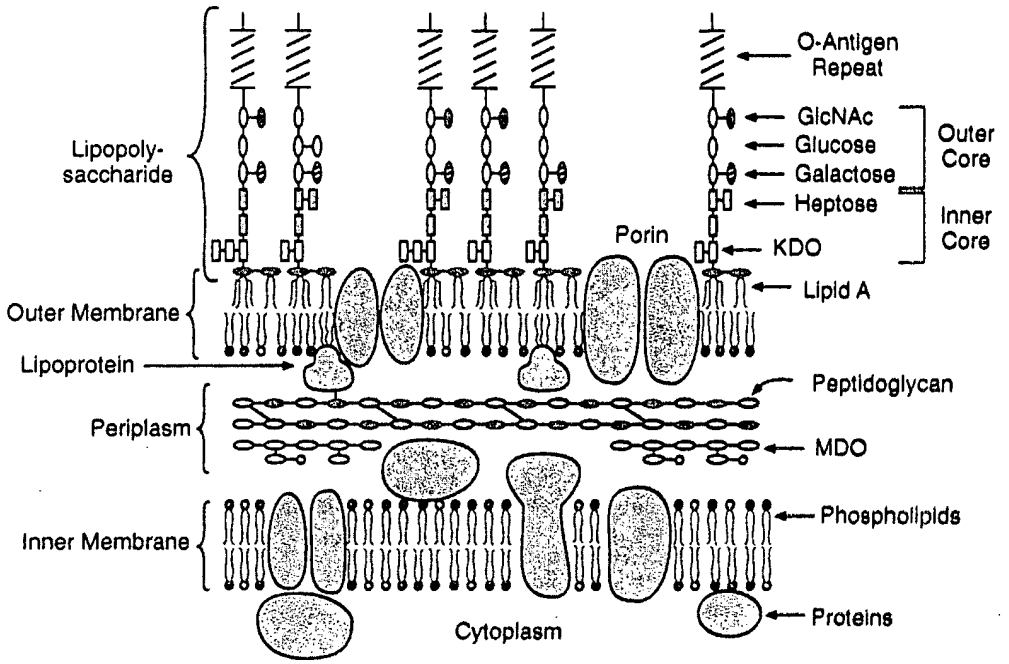


FIG. 1. Molecular representation of the envelope of a gram-negative bacterium. Ovals and rectangles represent sugar residues, whereas circles depict the polar head groups of glycerophospholipids (phosphatidylethanolamine in red and phosphatidylglycerol in yellow). MDO represents membrane-derived oligosaccharides (45). The core region shown is that of *E. coli* K-12 (36, 46), a strain that does not normally contain an O-antigen repeat unless transformed with an appropriate plasmid (36, 46).

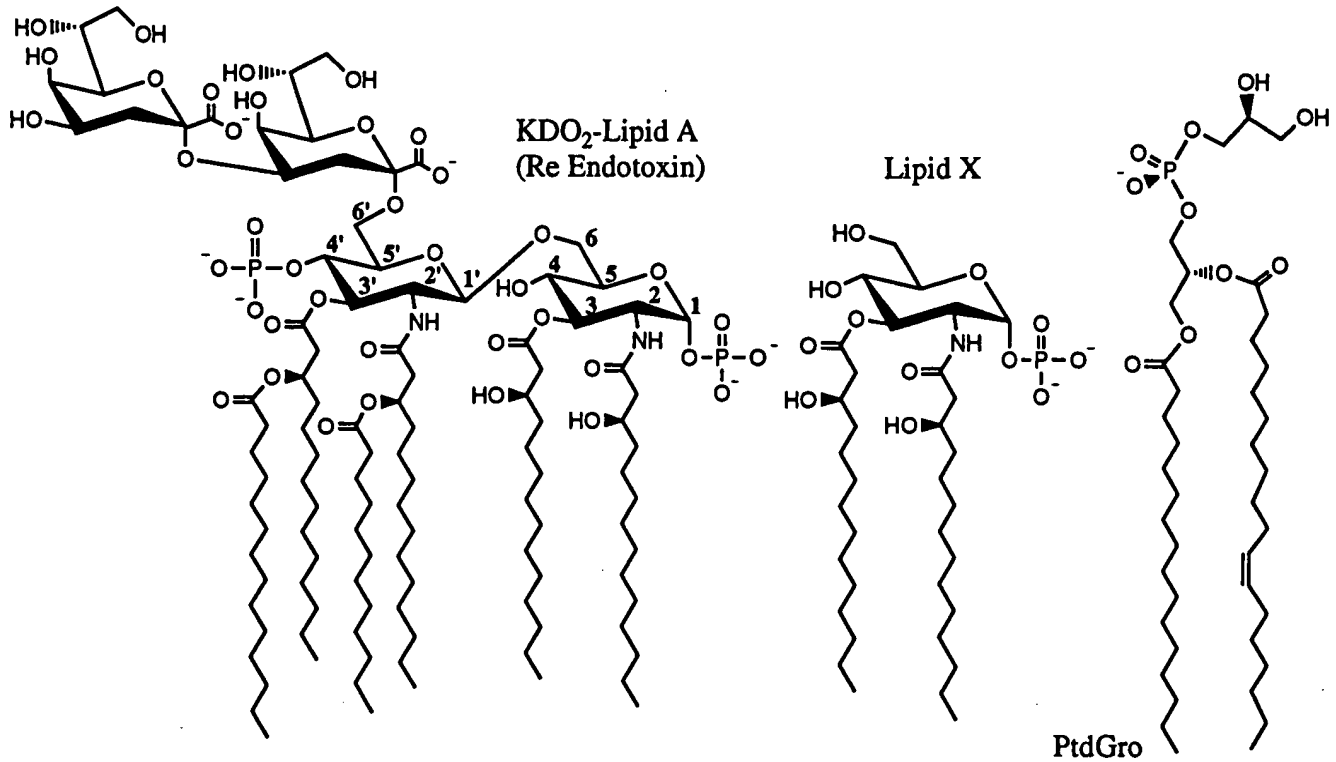


FIG. 2. Covalent structure of KDO_2-lipid A, the minimal endotoxin substructure required for growth. KDO_2-lipid A (Re endotoxin) can be isolated from heptose-deficient mutants of *E. coli* (36, 46). Lipid X (46) and phosphatidylglycerol (45) are drawn to scale. The standard numbering of the glucosamine carbons of lipid A (36, 46) is indicated. Re endotoxin is often modified with additional polar substituents (36, 46). PtdGro, phosphatidylglycerol.

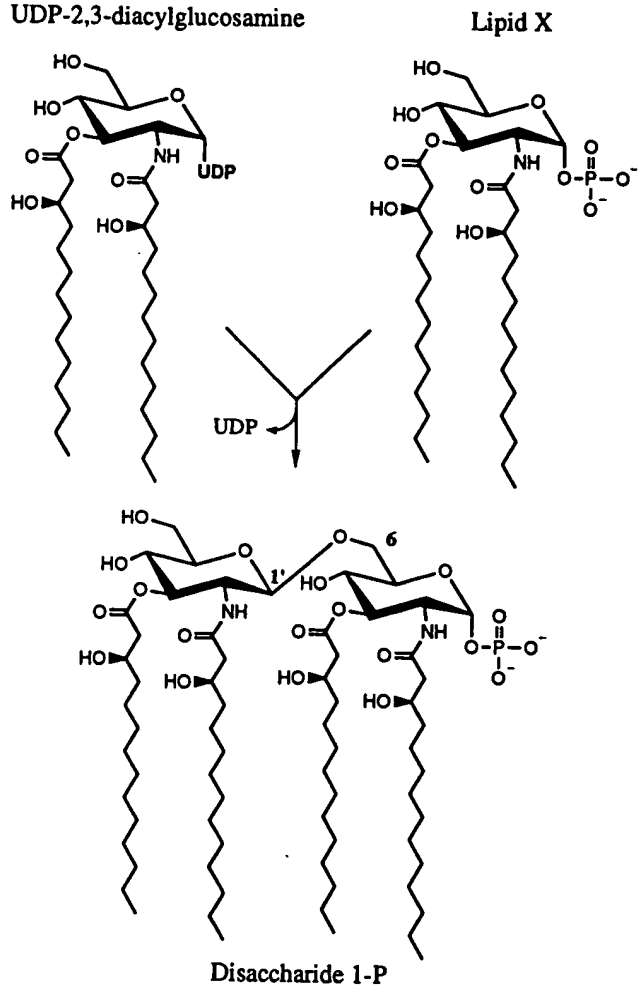


FIG. 3. Function of lipid X and UDP-2,3-diacylglucosamine in the enzymatic synthesis of the β, 1'-6 linkage of lipid A.

of UDP-GlcNAc (~0.01) is unfavorable (2), suggesting that *R*-3-hydroxymyristoyl-ACP (a thioester) may be more stable than UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcNAc (an oxygen ester). Whatever the chemical explanation, the significance of this observation is that deacetylation of UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcNAc (Fig. 4) (4) is the first irreversible step of lipid A biosynthesis.

Exposure of the glucosamine nitrogen by deacetylase permits incorporation of the *N*-linked *R*-3-hydroxymyristate to form UDP-2,3-diacylglucosamine (Fig. 4) (1, 3, 30). As with the *O*-acyltransferase, coenzyme A thioesters and myristoyl-ACP are not substrates (3, 30).

The key role of UDP-GlcNAc in lipid A biosynthesis shows that this nucleotide is situated at an important branch point in *E. coli*, as it is also a precursor of peptidoglycan (2). Similarly, *R*-3-hydroxymyristoyl-ACP can be used either in the biosynthesis of lipid A or in the generation of palmitate (33).

The demonstration of UDP-GlcNAc acyltransferases in all gram-negative bacteria so far examined has established the general importance of lipid X and UDP-2,3-diacylglucosamine as endotoxin precursors (65).

DISACCHARIDE FORMATION AND 4' KINASE

UDP-2,3-diacylglucosamine (Fig. 3) is the immediate precursor of the nonreducing sugar of lipid A (50). UDP-2,3-diacylglucosamine is also subject to cleavage at the pyrophosphate bond to generate 2,3-diacylglucosamine-1-phosphate (lipid X) (Fig. 5) (50). Disaccharide synthase transfers the 2,3-diacylglucosamine portion of UDP-2,3-diacylglucosamine to position 6 of lipid X (50) (Fig. 3 and 5). The disaccharide synthase cannot condense two molecules of UDP-2,3-diacylglucosamine directly, showing that the pyrophosphatase(s) that generates lipid X plays a key role (44, 50). The disaccharide synthase has been cloned, se-

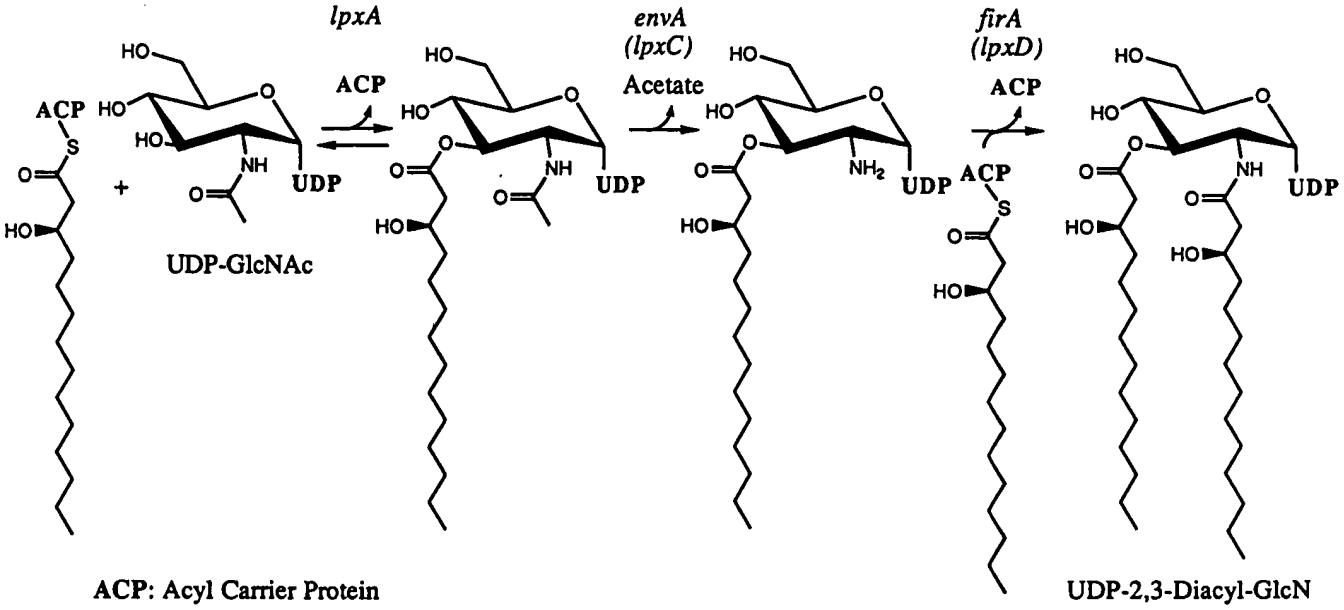
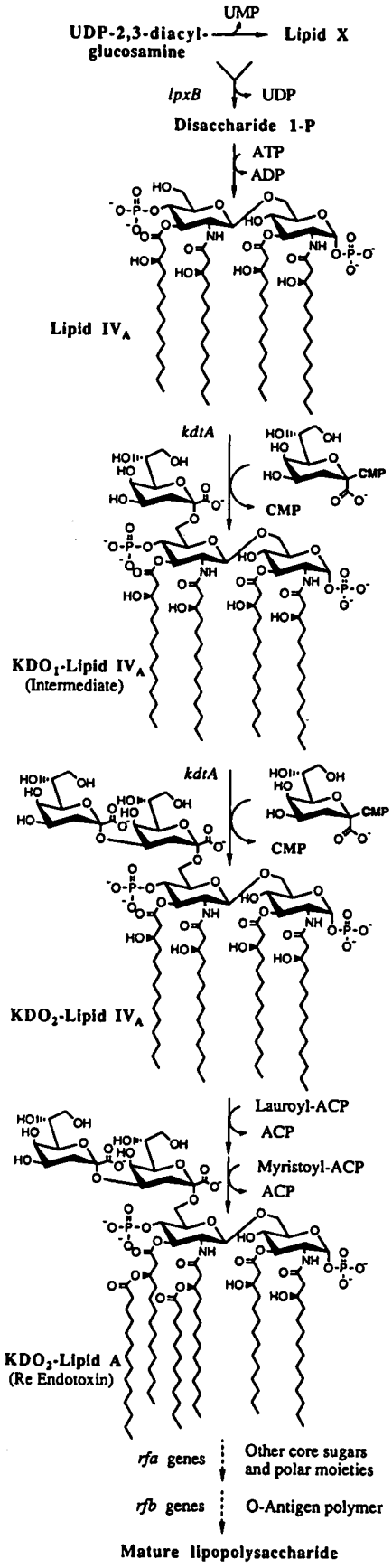


FIG. 4. Fatty acylation of UDP-GlcNAc in *E. coli*. The *lpxA*, *envA* (*lpxC*), and *firA* (*lpxD*) genes code for the enzymes indicated.



quenced, and purified (19, 44). This enzyme is useful for preparation of lipid A analogs (36, 44).

A specific kinase (51) incorporates the 4'-monophosphate, generating lipid IV$_A$ (Fig. 5). Lipid IV$_A$ also accumulates at 42°C in temperature-sensitive mutants defective in KDO biosynthesis (48, 52) or in cells treated with KDO biosynthesis inhibitors (25).

^{32}P-labeled lipid IV$_A$, prepared with this kinase (28), can be used for studying endotoxin binding to animal cells (27) or for detecting the distal enzymes of lipid A metabolism (12, 13) (Fig. 5). Unlike disaccharide synthase, this kinase is unstable and has not been cloned or purified (51). Since phosphorylation of the 4' position is thermodynamically favorable, access to pure 4' kinase would be useful for the synthesis of 1,4' disaccharide bis-phosphate analogs of lipid A. These analogs are known to include potent endotoxin antagonists (26, 31, 57) with potential clinical utility.

KDO TRANSFER AND LATE ACYLATION

Re LPS (Fig. 2) contains two distinct KDO residues, both derived from the labile nucleotide, CMP-KDO (46) (Fig. 5). The acceptor for the innermost KDO residue is the 6' OH of lipid IV$_A$ (Fig. 5), whereas the second KDO moiety is attached to the transient intermediate, KDO$_1$-lipid IV$_A$ (Fig. 5), at the 4 OH of the innermost KDO. KDO$_1$-lipid IV$_A$ accumulates to a small extent in enzymatic incubations at low KDO concentrations (8). Given the significant difference in the structures of the acceptors for the first and second KDO residues, it is remarkable that both KDO residues are incorporated by a bifunctional enzyme consisting of a single polypeptide (8) (Fig. 5). This enzyme is encoded by the *kdtA* gene (15) near min 81 on the *E. coli* chromosome, just clockwise of the *rfa* cluster. Overproduction of the *kdtA* gene product increases the specific activities of both the first and second KDO transferases in extracts (8, 15). The KDO transferase of *Chlamydia trachomatis*, which displays some sequence similarity to the *E. coli* enzyme, is a single polypeptide capable of incorporating at least three KDO residues (7).

Purified KDO transferase of *E. coli* (8, 12) recognizes lipid A disaccharide bis-phosphates as acceptors, but the extent of their acylation is not crucial. The presence of a 4' phosphate residue is an absolute requirement for substrate recognition (8, 12). This enzyme may share some common structural features with the serum LPS-binding protein (LBP) (59, 66), with lipid A recognizing components of the limulus clotting cascade (34, 37) or with putative endotoxin receptor(s) on animal cells (36, 46, 49). No sequence homologies between KDO transferase and eucaryotic lipid A-binding proteins have been discovered as yet.

Following attachment of KDO, additional acyltransferases complete the formation of lipid A by transferring laurate and myristate residues to KDO$_2$-lipid IV$_A$ (Fig. 5) (13). The resulting acyloxyacyl units are found in almost all lipid A molecules of diverse organisms (36, 46). The late acyltrans-

FIG. 5. Key role of UDP-2,3-diacylglucosamine in lipid A biosynthesis. The *lpxB* and *kdtA* genes code for the lipid A disaccharide synthase and KDO transferase, respectively. In wild-type cells, the levels of the intermediates in Fig. 4 and 5 are very low (less than 1,000 molecules per cell) (14).

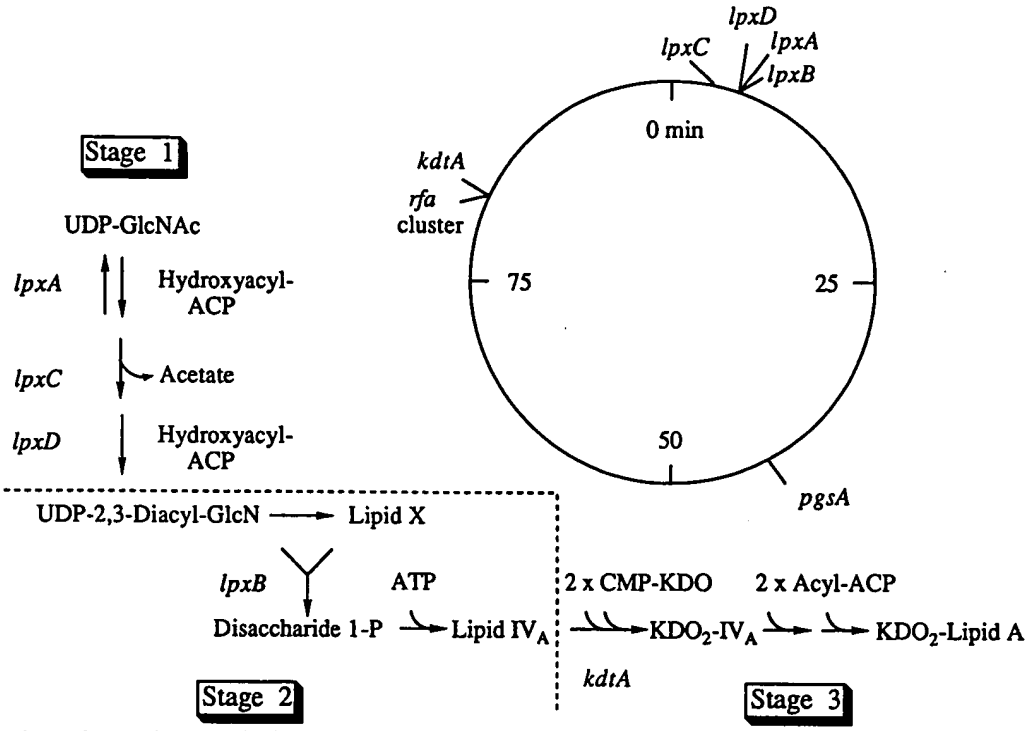


FIG. 6. Locations of genes involved in lipid A biosynthesis on the chromosome of *E. coli*. Other relevant genes, such as the *rfa* (15, 36, 55) cluster and *pgsA* (39, 40, 47), are also indicated. The pathway may be divided into three stages: stage 1, UDP-GlcNAc acylation; stage 2, disaccharide formation and 4′ kinase; and stage 3, KDO transfer and late acylation.

ferases of *E. coli* utilize thioesters of ACP as substrates (13). The late acyltransferases display an amazing dependence upon the KDO domain (13), accounting for the fact that KDO-deficent mutants accumulate lipid IV_A (25, 48, 52).

All of the lipid A synthetic enzymes are cytosolic or associated with the inner membrane (46). Novel transport functions needed for LPS export probably also exist but have not been defined.

MOLECULAR GENETICS OF LIPID A BIOSYNTHESIS

We have obtained genetic evidence that the lipid A biosynthesis pathway (Fig. 6) is the major one for lipid A generation in *E. coli* and is essential for cell viability (24). We first found that silent mutations in *pgsA* (encoding phosphatidylglycerophosphate synthase near min 42) (39, 40) were rendered conditionally lethal by otherwise silent, second site mutations in *lpxB*, the structural gene for the disaccharide synthase near min 4 (18, 39, 40). Cloning and sequencing of the disaccharide synthase (18, 19) revealed the presence of the *lpxA* gene, encoding UDP-GlcNAc *O*-acyltransferase, just upstream (Fig. 6) (17, 18). Characterization of temperature-sensitive mutations mapping near min 4 resulted in the isolation of SM101 (*lpxA2*), an organism with a defective UDP-GlcNAc *O*-acyltransferase and a 10-fold-reduced rate of lipid A synthesis in cells at 42°C (24). The properties of SM101 demonstrate the quantitative importance of this pathway (24). The rapid loss of viability of SM101 at 42°C suggests that inhibitors of these enzymes may be novel antibiotics (24).

The lipid A content of SM101 is 30% less than normal at the permissive temperature (30°C) (24). At 30°C, SM101 is hypersensitive to antibiotics that normally do not permeate the outer membrane, like rifampin and erythromycin (61).

In searching for other structural genes of lipid A synthesis, we examined all available rifampin-hypersensitive mutants. We found that deacetylase (Fig. 4) is encoded by the *envA* gene, located near min 2 on the *E. coli* chromosome (67). *envA* was first isolated and mapped in 1969 (41) as a mutation that causes antibiotic hypersensitivity and a delay in cell separation. Sequencing of *envA* (now designated *lpxC*) failed to reveal any similarity to genes of known function (6).

The specific activity of deacetylase is elevated 5- to 10-fold when lipid A synthesis is inhibited (2). How the deacetylase is up-regulated is unknown, but this observation is consistent with its function as the first committed step.

The *firA* gene of *E. coli* (now designated *lpxD*) (Fig. 6) was first defined as a second site mutation that caused reversal of rifampin resistance associated with certain mutations in the β subunit of RNA polymerase (21). Recently, the product of the *firA* gene (30) was identified as the *N*-acyltransferase of the lipid A pathway. The effects of *firA* mutations on rifampin resistance are presumably due to enhanced outer membrane permeability. The *firA* gene maps near *lpxA* and *lpxB* at min 4 (21). *firA* is identical to the *ssc* gene of *Salmonella typhimurium* (60). There is homology between the sequences of *firA* (*lpxD*) and *lpxA*, consistent with their functions (Fig. 4) (30, 60). The role of the operon(s) that includes *lpxA*, *lpxB*, and *lpxD* (17, 19, 30) requires further evaluation.

The only other structural gene that has been identified is *kdtA* (Fig. 6), encoding the KDO transferase (15). Colony autoradiography was used to obtain mutations in *kdtA* (15).

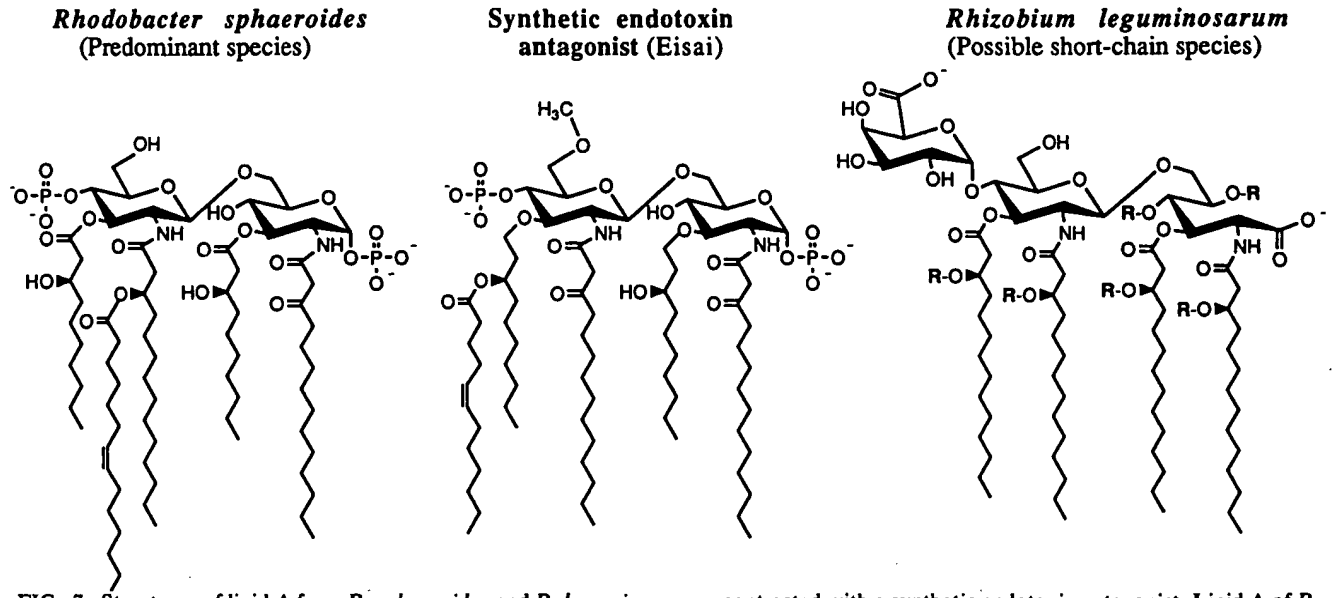


FIG. 7. Structures of lipid A from *R. sphaeroides* and *R. leguminosarum*, contrasted with a synthetic endotoxin antagonist. Lipid A of *R. sphaeroides* was one of the first endotoxin antagonists to be identified (43, 57). The ~100- to 1,000-fold-more-potent synthetic antagonist of the Eisai Co. was modeled on *Rhodobacter* lipid A. The Eisai compound is ~10-fold more active as an antagonist than *E. coli* LPS is as an agonist. The structure of lipid A from *R. leguminosarum* is not fully established (10, 11), and its biological activities are not yet known. Recent work by Carlson and Bhat (14a) indicates the presence of a 2-deoxy-2-aminogluconate residue in place of the reducing end glucosamine, as well as a 4′ galacturonic acid in place of phosphate. In addition to *R*-3-hydroxymyristate (as shown), *R. leguminosarum* lipid A contains 16-, 18-, and 28-carbon acyl chains (10, 11). R may be either an acyl residue or H.

LIPID A IN PHOTOSYNTHETIC BACTERIA AND RHIZOBIA

Examination of lipid A biosynthesis in *Rhodobacter sphaeroides* (43) and *Rhizobium leguminosarum* (10, 11) (Fig. 7) may provide some novel insights. Extracts of *R. sphaeroides* possess the UDP-GlcNAc acyltransferases (Fig. 4) (29a), but the origin of the unusual 3-keto-myristate residue (Fig. 7) (43) is uncertain. Lipid A from *R. sphaeroides* is interesting, in that it is an antagonist of the action of *E. coli* lipid A on animal cells (26, 57). More-potent, synthetic endotoxin antagonists (Fig. 7) modeled on *Rhodobacter* lipid A have recently been reported (14b).

Lipid A from *R. leguminosarum* lacks phosphate altogether and does not contain a glucosamine disaccharide (10, 11). Carlson and Bhat (14a) have proposed a structure like that in Fig. 7, in which carboxyl groups seem to function as surrogates of the phosphates that are usually attached at positions 1 and 4′. *R. leguminosarum* lipid A contains a 2-deoxy-2-aminogluconate residue in place of the glucosamine 1-phosphate at the reducing end, and it bears a galacturonic acid moiety instead of a monophosphate at position 4′. We have detected the UDP-GlcNAc acyltransferases (Fig. 4) (29a) in extracts of *R. leguminosarum*. It will be interesting to determine whether *R. leguminosarum* also contains a disaccharide synthase, a 4′ kinase, etc., and how the 2-deoxy-2-aminogluconate is made. Another feature of *R. leguminosarum* is the presence of very long (28-carbon) acyl chains (11). The unique structure of lipid A in *R. leguminosarum* may reflect functional roles in symbiosis.

OVERVIEW OF ENDOTOXIN PHARMACOLOGY

Considerable progress has been made recently in defining the mechanisms by which endotoxins (lipid A) interact with animal cells (Fig. 8). During infections, LPS can dissociate from bacteria (or membrane fragments) and bind to LBP (59). LBP may function as a lipid transfer protein that delivers LPS to CD14 (59, 66). CD14 is a surface protein of macrophages and other LPS-responsive cells (55).

Discovery of the scheme in Fig. 8 was based on the effects of antibodies directed against macrophage surface proteins on the LPS response and on the LBP requirement for optimal LPS stimulation (59, 66). Certain cells, such as 70Z/3 pre-B lymphocytes, lack CD14 (32). Although these cells can respond to high levels (nanomolar) of lipid A (32, 49), they respond to low levels (picomolar) when transfected with CD14 (32).

CD14 is bound to the cell surface by a phosphatidylinositol anchor (55). CD14 may direct LPS to another, as yet unknown, transmembrane protein that generates intracellular signal(s). In macrophages, the signals resulting from this association stimulate transcription of mRNA encoding cytokines, like tumor necrosis factor and interleukin-1 (9, 22). Excessive production of these proteins (not lipid A itself) is responsible for the symptoms of endotoxin-induced shock (9, 22, 35).

The model shown in Fig. 8 bears some resemblance to what is known about receptors for interleukin-6 (56). The relevant intracellular signals resulting from LPS stimulation remain to be defined, but rapid activation of tyrosine phosphorylation and MAP kinase(s) is known to occur (63).

Lipid A and LPS are also bound and internalized by cells that express the macrophage scavenger receptor (27; not shown in Fig. 8). Inhibition of uptake mediated by the scavenger receptor does not prevent stimulation of cytokine synthesis (27), indicating that the scavenger receptor is not involved in signal transduction.

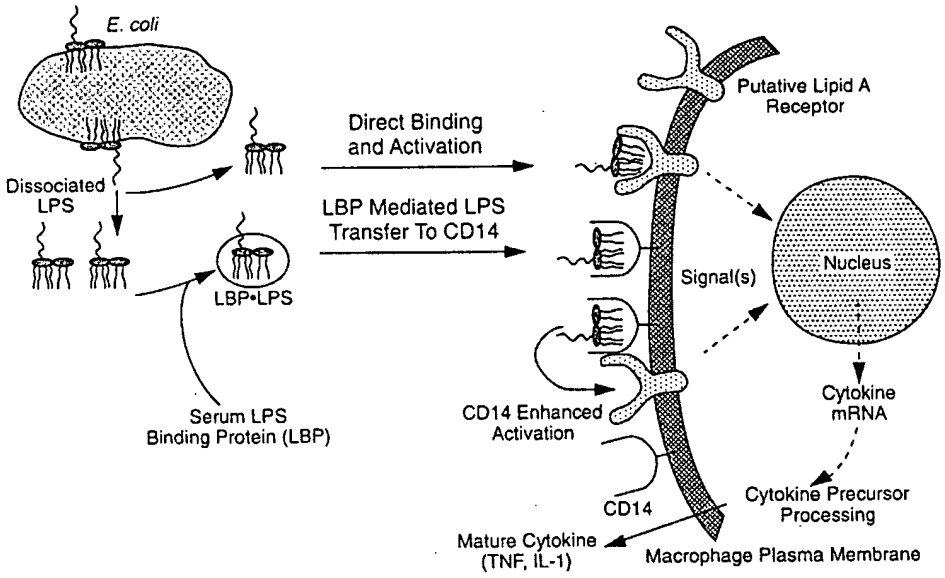


FIG. 8. Role of CD14 and LPS-binding protein in the activation of macrophages by endotoxins. This model is based on the studies of Ulevitch and Wright and coworkers (32, 49, 59, 66). The identity of the putative transmembrane protein downstream of CD14 is still unknown, but binding of LPS to CD14 has recently been demonstrated (59a). The functioning of LBP as a transfer protein for lipid A is suggested by its sequence similarity to cholesteryl ester transfer protein (23). TNF, tumor necrosis factor; IL-1, interleukin-1.

APPROACHES TO THE TREATMENT OF ENDOTOXIN-INDUCED SHOCK

Complications of gram-negative infections are a common cause of death in debilitated patients (42). On the basis of current knowledge of the pathophysiology of endotoxemia (Fig. 8), three general approaches are being explored (20). The first involves sequestration of lipid A with antibodies (62) or with a bacteriocidal protein of neutrophils (64). Clinical trials with the former have been disappointing (20, 62), and results with bacteriocidal protein are not yet available. The second approach involves blocking of the action of key cytokines, as with antibodies to tumor necrosis factor (16) or with a protein antagonist that binds to the interleukin-1 receptor (5, 20). Despite initial promise, phase III trials in patients have failed to confirm good efficacy (5, 20). The newest approach (not yet tested in patients) involves lipid A antagonists, such as analogs of *R. sphaeroides* lipid A (26, 57) (Fig. 7), to block LPS activation of macrophages (Fig. 8). Blocking the initial event(s) should prevent the production of all mediators.

The difficulties encountered so far (20, 62) in developing a therapy for endotoxin-induced shock will eventually be overcome. The enormous progress that has been made since 1983 in unravelling the chemistry and biology of bacterial endotoxins (36, 46, 53) has yielded a wealth of new approaches that remain to be tested.

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